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(57) Abstract

The invention provides methods of transducing non–endothelial cardiovascular cells with a recombinant Togavirus vector, vector kits, and vectors.

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SEMLIKI FOREST VIRUS VECTORS FOR GENE TRANSFER INTO NON-ENDOTHELIAL CARDIOVASCULAR CELLS

FIELD OF THE INVENTION

The invention provides methods of transducing non-endothelial cardiovascular cells with a recombinant Togavirus vector, vectors, vector kits, and transduced cells.

BACKGROUND

Somatic gene transfer, also known as gene therapy, is currently being evaluated as a treatment for a variety of diseases. In cardiovascular disorders, somatic gene transfer methods have been studied for treatment of vessel narrowing (stenosis or restenosis) caused, for example, by mechanical injury due to balloon angioplasty and atherosclerotic plaques (Lucas *et al.*, *Circulation* 94:2890-2900 (1996)). Restenosis after therapeutic angioplasty is typically caused by local proliferation of smooth muscle cells, triggered by platelet adhesion to freshly dilated vessel surfaces. This type of restenosis is seen in approximately 20-30% of patients within 6 months of angioplasty (*see, e.g.*, *Harrison's Principles of Internal Medicine* (Isselbacher *et al.* eds., 13th ed. 1994)).

The success of gene therapy techniques for treatment of restenosis will largely depend on efficiency of gene transfer, timing, amount of gene expression, and in

particular, specificity for cell type. Vector specificity for cell type is important for treatment of restenosis, since the goal is specific inhibition of smooth muscle hyperplasia. Other cell types present at the site of the restenosis should be maintained, e.g., endothelial and endocardial cells.

Several viral vectors have been shown to reduce smooth muscle proliferation both *in vitro* and *in vivo* (Feldman *et al.*, *Cardiovasc. Res.* 32:194-207). Replication-deficient adenovirus is a widely used carrier for somatic gene transfer. Such adenovirus vectors are more efficient than retroviral and non-viral vectors and are regarded as an effective system for gene transfer. However, the drawbacks of these vectors include lack of specificity for cell type, immunogenicity in the host, and late onset of transgene expression. Accordingly, vectors that specifically transduce smooth muscle cells would be desirable.

SUMMARY OF THE INVENTION

The present invention provides methods of selectively transducing non-endothelial cells such as vascular smooth muscle cells using Togavirus or Togavirus-derived vectors. These vectors have the ability specifically to transduce the cardiovascular cells that cause restenosis and deliver a target nucleic acid that inhibits cell growth or viability, thereby providing an effective therapy for restenosis.

In one aspect, the invention provides a method of transducing a selected cell with a target nucleic acid, by contacting the selected cell with a vector. The vector has a vector nucleic acid encoding the target nucleic acid and the selected cell is a non-endothelial cardiovascular cell.

In one embodiment, the selected cell is a human cell. In another embodiment, transduction of the selected cell is performed *in vivo*, preferably following balloon angioplasty. In a further embodiment, transduction of the non-endothelial cardiovascular cells inhibit restenosis after balloon angioplasty. In yet another embodiment, the vector is introduced into a patient by local injection at a site in the patient proximal to the selected cell. In another embodiment, the vector is a Togavirus and the vector nucleic acid is a Togavirus packageable nucleic acid.

In one embodiment the vector has a p62 protein that is cleaved *in vitro* by chymotrypsin to produce a mature E2 protein.

In another aspect the invention provides a method of preferentially transducing selected cells with a target nucleic acid *in vivo*, by contacting the selected cells and further contacting non-selected cells with a recombinant Togavirus vector. The Togavirus vector has a target nucleic acid. In this method, the selected cells are preferentially transduced by the Togavirus vector as compared to the non-selected cells.

In another embodiment, the selected cells are non-endothelial cardiovascular cells and the non-selected cells are endothelial cardiovascular cells. In a further embodiment, the non-endothelial cardiovascular cells are transduced with an efficiency of at least about 50 times the efficiency of transducing the non-endothelial cardiovascular cells. In another embodiment, the vector is a Togavirus and the nucleic acid is a Togavirus packageable nucleic acid.

In another aspect, the invention provides an assay for measuring the relative transduction efficiency of a Togavirus vector for a first test cell as compared to a second test cell. The Togavirus vector has a vector nucleic acid. The method includes the following steps: (1) contacting the first test cell with the Togavirus vector; (2) contacting the second test cell with the Togavirus vector; (3) measuring the level of vector nucleic acid in the first and second test cell; and (4) comparing the level of vector nucleic acid in the first and second cell. This comparison provides a determination of the relative transduction efficiency of the Togavirus vector in the first and second test cells.

In another embodiment, the level of vector nucleic acid is measured indirectly by measuring the level of expression of an encoded nucleic acid or by measuring the level of an encoded peptide. In yet another embodiment, the first test cell is a non-endothelial cardiovascular cell and the second test cell is an endothelial cardiovascular cell. In another embodiment, the vector is a Togavirus and the vector nucleic acid is a Togavirus packageable nucleic acid.

In another aspect; the invention provides a non-endothelial cardiovascular cell that has a vector nucleic acid encoding a target nucleic acid.

In one embodiment, the non-endothelial cardiovascular cell is *in vivo* or *in vitro*. In another embodiment, the vector nucleic acid is a Togavirus-packageable nucleic acid.

In another aspect, the invention provides a Togavirus vector having a nucleic acid. The vector nucleic acid has a target nucleic acid subsequence that encodes a

restenosis inhibitor. In one embodiment, the vector is a Togavirus and the nucleic acid is a Togavirus packageable nucleic acid.

In another aspect, the invention provides a kit having a container, a vector that has a vector nucleic acid, and instructions for practicing the method described above for transducing a selected cell with a target nucleic acid.

For all of the aspects described above, in one embodiment, the vector is a Semliki Forest virus, a Togavirus particle, or a Semliki Forest virus particle. For all of the aspects described above, in one embodiment, the non-endothelial cardiovascular cell is a vascular smooth muscle cell or a cardiomyocyte.

For all of the aspects described above, in one embodiment, the target nucleic acid encodes a restenosis inhibitor. For all of the aspects described above, in one embodiment, the target nucleic acid encodes a restenosis inhibitor selected from the group consisting of a ribozyme, an antisense RNA, a suicide protein, and a transdominant inhibitor. For all of the aspects described above, in one embodiment, the target nucleic acid encodes a restenosis inhibitor selected from the group consisting of a c-myc inhibitor, a c-myb inhibitor, an angiotensin converting enzyme (ACE) inhibitor, a FGF inhibitor, a PDGF inhibitor, and a TGF-β inhibitor. For all of the aspects described above, in one embodiment, the target nucleic acid encodes a restenosis inhibitor that is herpes simplex virus thymidine kinase.

BRIEF DESCRIPTION OF THE DRAWING

Figure 1 illustrates selective expression in denuded rat aorta of a luciferase marker gene carried by an SFV vector.

Figures 2A and 2B illustrate the clearcut blue staining that was observed in multiple cells per section, β-galactosidase expression was confined to the vascular smooth muscle cells.

DETAILED DESCRIPTION

I. Introduction

This invention provides an efficient approach to gene therapy for treatment of restenosis that is based on a Togavirus vector. This vector selectively transduces non-endothelial cells, such as vascular smooth muscle cells, and delivers a target nucleic acid,

which inhibits cell growth and or viability. The ability of Togaviruses such as Semliki Forest virus ("SFV"), and vectors derived from such Togaviruses, to rapidly and efficiently transfer target nucleic acids into non-endothelial cardiovascular cells is particularly important for treatment of vascular lesions, e.g., those caused by atherosclerotic plaques or mechanical injury such as balloon angioplasty and other related techniques. In such treatment, the endothelium and endocardium must be protected, whereas the vascular smooth muscle cells that form lesions are specifically targeted. Furthermore, the Togavirus vector of the invention is particularly suitable for blocking early gene expression, thereby preventing a cascade of molecular processes that lead to deleterious processes such as restenosis.

II. Definitions

As used herein, the following terms have the meanings ascribed to them unless specified otherwise.

A virus or vector "transduces" a cell when it transfers nucleic acid into the cell. Transduction can occur by any means that allows the nucleic acid to enter the cell, e.g., infection, transformation, or other means of transport across the cell membrane.

"Selected cell" refers to an identified subset of cells for which specific vector transduction is desired, in order to obtain delivery of the target nucleic acid to the cell. For example, non-endothelial cardiovascular cells are a subset of cardiovascular cells that a vector such as an SFV vector specifically transduces for delivery of a target nucleic acid to the selected cell.

"Preferentially transduced" refers to a subset of cells that are transducible by a vector with a higher efficiency of transduction than a nonselected subset of cells.

"Efficiency of transduction" refers to the relative ability of a vector to transduce a selected cell as compared to the ability of the vector to transduce a control cell. The efficiency of transduction for a selected cells is at least twice that of the control cell, preferably 10-50 times the efficiency of transduction for the control cell.

"Vector" refers to a composition which can transduce, transform or infect a cell, thereby causing the cell to express vector-encoded nucleic acids and, optionally, proteins other than those native to the cell, or in a manner not native to the cell. A vector includes a nucleic acid (ordinarily RNA or DNA) to be expressed by the cell (a

"vector nucleic acid"). The vector nucleic acid optionally includes a viral packaging site, which allows the vector nucleic acid to be packaged into a viral particle. The vector nucleic acid may comprise a "target nucleic acid." A vector optionally includes materials to aid in achieving entry of the nucleic acid into the cell, such as a Togavirus particle, liposome, protein coating or the like.

"Togavirus particle" refers to a virus particle from the Togavirus family that has the ability to transduce a cell. Generally, the Togavirus particle includes a Togavirus-packageable vector nucleic acid, which may further encode a target nucleic acid. The Togavirus particle delivers the target nucleic acid to the cell via transduction. The Togavirus particle may be formed via trans-complementation using packaging cells.

"Semliki Forest" virus (SFV) is a virus species, which is a member of the "Alphavirus" genus, one of two genera of the viral family *Togaviridae* (Togavirus). Other members of the Alphavirus genus include, e.g., Sindbis virus, Ross River virus, and Eastern and Western equine encephalitis viruses. Preferred vectors of the invention are members are Alphaviruses.

A "vector nucleic acid" refers to the nucleic acid that is associated with the vector. The vector nucleic acid can comprise a target nucleic acid and/or a Togavirus packageable nucleic acid.

"Target nucleic acid" refers to a nucleic acid that is included in the vector for delivery to a selected cell via transduction. The target nucleic acid is any nucleic acid suitable for delivery to the cell, e.g., a nucleic acid encoding a protein, an antisense nucleic acid, or a ribozyme. The target nucleic acid may include a promoter. The target nucleic acid may have the ability to integrate into the transduced cell genome, or it may maintain a circular or linear episomal state in the transduced cell. The target nucleic acid may consist of SFV sequences or heterologous sequences.

"Togavirus-packageable vector nucleic acid" refers to a vector nucleic acid that includes a packaging site from a Togavirus, e.g., an SFV packaging site. The presence of the packaging site allows the vector nucleic acid to be assembled into a Togavirus particle for transduction of a selected cell. Thus, a "packaging sequence" or "packaging signal" is a viral nucleic acid sequence that directs efficient and specific encapsidation of the nucleic acid that includes this site into viral particles. An example of a Togavirus packaging signal is nucleotides 1-247 of the SFV genome.

"Endothelial cardiovascular cell" refers to a cell type that makes up the tissue that lines blood vessels (the endothelium) and the heart (the endocardium). The endothelium is a single layer of closely arrayed, flat cells that provides a barrier between the lumen of the vessel or heart and the underlying muscle cells.

"Non-endothelial cardiovascular cell" refers in general to those cells of the cardiovascular system (blood vessels and heart) that are not part of the endothelium or the endocardium. Non-endothelial cardiovascular cells include, e.g., vascular smooth muscle cells and cardiac muscle cells.

"Vascular smooth muscle" or "smooth muscle" refer to a non-endothelial cell that makes up the muscle component of blood vessels.

"Cardiomyocyte" or "cardiac muscle cell" refer to a non-endothelial cell that forms the heart muscle.

"Restenosis" refers in general to a reoccurrence of a narrowing (stenosis) or occlusion of a blood vessel.

"Restenosis inhibitor" refers to any substance such as a protein, antisense nucleic acid, or ribozyme that can be used directly or indirectly to inhibit processes, e.g., smooth muscle proliferation, that lead to restenosis. Examples of restenosis inhibitors include, e.g., antisense nucleic acids or ribozymes that inhibit targets such as c-myc RNA or non-endothelial cell growth factor mRNAs (PDGF, FGF-1); transdominant inhibitors of non-endothelial cell proteins such as angiotensin converting enzyme (ACE) and cell cycle control proteins; suicide genes such as thymidine kinase that locally convert chemicals such as ganciclovir to cellular toxins for dividing non-endothelial cells; and proteins that enhance re-endothelialization such as certain growth factors (VEGF).

"Balloon angioplasty" refers to a technique for revascularizing a vessel that has a stenosis or occlusion. The term is also intended to generally encompass related non-balloon angioplasty techniques, such as laser techniques, intravascular stents, and mechanical atherectomy. "Atherosclerotic plaque" refers to a blood vessel lesion that leads to stenosis or occlusion of the vessel.

"C-myc" and "c-myc RNA" refer respectively to the gene encoding c-myc and its resulting transcript. C-myc is a cellular proto-oncogene that is likely involved in cell growth control and mitogenic response.

"p62 protein" refers to a spike glycoprotein that is a structural protein encoded by the SFV genome and is involved in viral infection of host cells. The p62 protein forms a dimer with the E1 protein. The p62 protein is cleaved by a protease to its mature form, the "E2 protein," or the "mature E2 protein" (Berglund *et al.*, *Biotechnology* 11:916-920 (1993)).

The term "nucleic acid" refers to a deoxyribonucleotide or ribonucleotide polymer in either single- or double-stranded form, and unless otherwise limited, encompasses known analogues of natural nucleotides that hybridize to nucleic acids in manner similar to naturally occurring nucleotides. Unless otherwise indicated, a particular nucleic acid sequence optionally includes the complementary sequence thereof.

The term "subsequence" in the context of a particular nucleic acid sequence refers to a region of the nucleic acid equal to or smaller than the specified nucleic acid.

A "ribozyme" refers to a catalytic RNA molecule that cleaves a target RNA through ribonuclease activity.

III. Making Togavirus vectors and Togavirus-packageable vector nucleic acids

A. The Togavirus vector system

Togaviridae is a family of viruses whose genome consists of a single positive strand of RNA. Togaviruses typically produce a lytic infection in the host cell. A unique feature of these viruses is that they replicate in the cell cytoplasm as RNA, without using DNA intermediate. Replicase is the viral RNA polymerase that recognizes Togavirus promoters (genomic and subgenomic promoters) and uses the viral RNA genome as a template to transcribe/amplify viral RNA. The *Togaviridae* genomic RNA thus serves a dual role. First, it is the messenger RNA for translation of viral replicase. Second, the genomic plus-strand RNA also serves as a template for synthesis of a minus-strand RNA by the replicase proteins, which recognize specific viral promoters.

In the wild type virus, the resulting minus-strand RNA is further transcribed by replicase into two additional RNAs: a genomic plus-strand RNA and a smaller RNA that encodes structural proteins for nucleocapsid formation. The smaller RNA is amplified using a "subgenomic" replicase promoter. (Schlesinger & Schlesinger, *Togaviridae: The Viruses and Their Replication*, in *Fields Virology*, pp. 825-841 (Fields *et al.*, eds., 3d ed. 1996).

The "replicase gene cluster" is a group of four nonstructural proteins (NSP 1-4) from the *Togaviridae* viral family that encode the proteins required for replicase enzyme activity. The replicase proteins include the replicative activities required to transcribe minus-strand RNA, which is then used by the replicase proteins as a template to produce new plus-strand genomic RNA or shorter, "26S" RNA that encodes structural proteins.

"Replicase promoter," "genomic promoter," and "subgenomic promoter" refer to promoters from the *Togaviridae* viral family. In the wild type virus, a replicase promoter is used by the replicase proteins to transcribe full length minus strands from the positive strand genome. This full length minus strand in turn serves as a template for two transcripts. The first transcript is a shorter "26S" RNA, which represents about 1/3 of the viral genome and encodes structural proteins required for virus particle assembly. The 26S RNA is transcribed by replicase proteins, using a subgenomic promoter. The second transcript is the positive strand viral genome. The terms replicase-recognized, genomic, and subgenomic promoters thus refer to viral promoters recognized by replicase proteins.

The efficient RNA transcription/amplification activity of replicase in wild type Togavirus ensures that practically all of the ribosomes of the host cell will be involved in synthesis of viral encoded proteins. The subgenomic promoter is a strong promoter that drives the production of large amounts of the subgenomic transcript, which encodes the structural proteins of the virus. Because SFV replication occurs in the cytoplasm, it is a convenient system for expression of heterologous nucleic acid that obviates problems of splicing, capping, and transport found in other systems. The strength of the subgenomic promoter also makes it an attractive candidate for driving transcription of a heterologous nucleic acid.

Togavirus vectors of the invention are derived from Togaviruses such as SFV or Sindbis virus. Preferably, the vectors are derived from the genus Alphavirus. In one embodiment, the vector includes the following elements: (1) a Togavirus-packageable vector nucleic acid with a packaging site, replication sites, and a Togavirus promoter; (2) a Togavirus promoter (often the subgenomic promoter) operably linked to a target nucleic acid; (3) the genes encoding NSP 1-4 ("replicase"); and (4) structural proteins that make up a nucleocapsid.

In another embodiment, the Togavirus-derived vector retains only the structural proteins of the nucleocapsid required for cell tropism, so that the vector retains the ability to transduce selected cells such as vascular smooth muscle cells. Such Togavirus-derived vectors can include, e.g., liposomes that have Togavirus nucleocapsid proteins and that include vector nucleic acids, *see, e.g.*, USSN 08/454,641. In such a case, the vector nucleic acid does not require a Togavirus packaging site. Such a vector nucleic acid may optionally have a replication site, a Togavirus promoter, and the genes encoding NSP 1-4.

The Togavirus vector nucleic acid that includes the elements 1-3 described above can be packaged via trans-complementation with element 4 described above. SFV provides a well known example of production of a Togavirus vector via trans-complementation (*see, e.g.*, Liljeström & Garoff, *Biotechnology* 9:1356-1361 (1991); *Current Protocols in Molecular Biology* (Ausubel *et al.*, eds., 1994)). SFV vectors are commercially available or can be created *de novo* using recombinant methodology. Vector nucleic acids that do not require a Togavirus packaging site are packaged into vectors by standard methodology, e.g., by forming a lipid:nucleic acid complex, and transduced according to standard methodology, e.g., liposome transduction *see, e.g.*, USSN 08/454,641, herein incorporated by reference in its entirety.

In one embodiment, an SFV-packageable vector nucleic acid or the vector nucleic acid is engineered to contain the target nucleic acid of interest operably linked to the SFV subgenomic promoter. The target nucleic acid replaces the SFV structural genes to create a replication-deficient viral genome. The SFV-packageable vector nucleic acid typically retains the genes encoding replicase (NSP 1-4). The SFV-packageable vector also includes the regions required in cis for packaging and replication (nucleotides 1-247 and nucleotides 11423-11,441 of the viral genome; *see* Liljeström & Garoff, *Biotechnology* 9:1356-1361 (1991)).

The SFV-packageable vector nucleic acid is then introduced into packaging cells, as described below. The SFV-packageable vector nucleic acid is either introduced as DNA and then transcribed in the packaging cell or is introduced to the cell as RNA. Replicase proteins are translated from the SFV-packageable vector RNA in the packaging cell and in turn transcribe the vector nucleic acid, which acts as the viral genome. The SFV-packageable vector RNA is then complemented by packaging cells that provide the

structural capsid proteins in trans. The capsid protein recognizes a packaging site on the SFV-packageable RNA to package the RNA into a viral particle. Thus, a viral particle is created that contains a recombinant SFV-packageable vector RNA, which contains the target nucleic acid of choice. Further details regarding the construction of packaging cells and production of the viral vector are provided below.

Once the SFV particle (which is typically replication deficient) is delivered to the cell of choice via transduction, replicase is translated from the RNA, and then the replicase transcribes and amplifies the target nucleic acid using an SFV promoter that is recognized by replicase.

Vectors that do not encode a complete set of viral packaging components (e.g., structural proteins) are "replication deficient." These vectors are "trans-rescuable" when the vectors are packaged by viral proteins supplied by trans-complementation in a packaging cell. If an SFV-packageable nucleic acid is used to transform a cell infected with SFV in a cell culture or organism infected with SFV, the SFV-packageable nucleic acid will be replicated and disseminated throughout the organism in concert with the infecting SFV virus particle. However, the SFV-packageable nucleic acid is *not* itself able to produce further virus particles, because packaging functions are supplied by the infective SFV virus via trans-complementation. Thus, the SFV-packageable nucleic acid packaged in the virus particle undergoes only one round of transduction/infection.

B. Construction of a recombinant vector nucleic acid

In the present invention, recombinant DNA plasmids are constructed that can be used to make Togavirus vectors. In one embodiment, the DNA plasmid includes nucleic acids that encode the typical elements of the Togavirus-packageable nucleic acid described above: (1) a Togavirus-packageable vector nucleic acid with a packaging site, replication sites and a Togavirus promoter recognized by replicase; (2) a Togavirus subgenomic (replicase-recognized) promoter operably linked to a target nucleic acid; and (3) the genes encoding NSP 1-4. As described above, in another embodiment, vector nucleic acids do not require a Togavirus packaging site, and that optionally include Togavirus promoters and NSP 1-4 genes. DNA plasmids that are used to create packaging cells to provide viral structural proteins and trans-complement the Togavirus-packageable vector nucleic acid can also be constructed using the following methods (*see*

generally Sambrook *et al. Molecular Cloning-A Laboratory Manual* Vol. 1-3 (2nd ed. 1989); Ausubel *et al., supra*).

A recombinant DNA plasmid used to make a Togavirus vector is prepared by first isolating the constituent nucleic acids. The nucleic acids are then joined, for example, using restriction endonuclease sites at the ends of the molecule. The recombinant molecule is ligated into suitable a plasmid or vector. Methods for preparing a recombinant nucleic acid are know by those skilled in the art (*see* Sambrook *et al.*, *Molecular Cloning. A Laboratory Manual* (2d ed. 1989); Ausubel *et al.*, *Current Protocols in Molecular Biology* (1995)).

The term "recombinant" when used with reference to a cell indicates that the cell replicates or expresses a nucleic acid, or expresses a peptide or protein encoded by nucleic acid whose origin is exogenous to the cell. Recombinant cells can express genes that are not found within the native (non-recombinant) form of the cell. Recombinant cells can also express genes found in the native form of the cell wherein the genes are re-introduced into the cell by artificial means.

A "recombinant expression cassette" or simply an "expression cassette" is a nucleic acid construct, generated recombinantly or synthetically, with nucleic acid elements which permit transcription of a particular nucleic acid in a cell. The recombinant expression cassette can be part of a plasmid, virus, nucleic acid fragment, or vector nucleic acid. Typically, the recombinant expression cassette includes a nucleic acid to be transcribed (typically the target nucleic acid), and a promoter. In some embodiments, the expression cassette also includes, e.g., an origin of replication.

The term "heterologous" when used with reference to portions of a nucleic acid indicates that the nucleic acid comprises two or more subsequences which are not found in the same relationship to each other in nature. For instance, the nucleic acid is typically recombinantly produced, having two or more sequences from unrelated genes arranged to make a new functional nucleic acid.

One preferred method for obtaining specific nucleic acids combines the use of synthetic oligonucleotide primers and amplification of a target mRNA or DNA template via polymerase chain reaction ("PCR"), reverse transcription ("RT") or ligase chain reaction ("LCR") (*see* U.S. Patents 4,683,195 and 4,683,202). Restriction endonuclease sites can be incorporated into the primers. Genes amplified, e.g., by PCR

can be purified from agarose gels and ligated together. Alterations in the natural gene sequence can be introduced by techniques such as *in vitro* mutagenesis and PCR using primers that have been designed to incorporate appropriate mutations. Another preferred method uses known restriction endonuclease sites to isolate nucleic acid fragments from DNA plasmids.

A "promoter" is an array of nucleic acid control sequences which direct transcription of a nucleic acid. As used herein, a promoter includes necessary nucleic acid sequences near the start site of transcription, such as, in the case of a polymerase II type promoter, a TATA element. A promoter also optionally includes distal enhancer or repressor elements which can be located as much as several thousand base pairs from the start site of transcription. A "constitutive" promoter is a promoter which is active under most environmental and developmental conditions. An "inducible" promoter is a promoter which is under environmental or developmental regulation. The term "operably linked" refers to a functional linkage between a nucleic acid expression control sequence (such as a promoter, or array of transcription factor binding sites) and a second nucleic acid sequence, wherein the expression control sequence directs transcription of the nucleic acid corresponding to the second sequence.

The Togavirus-packageable vector nucleic acid or vector nucleic acid includes promoters operably linked to a nucleic acid sequence. Such promoters can either be promoters intended to promote transcription of the target nucleic acid after transduction of the Togavirus vector into the target cell, or intended to transcribe the Togavirus-packageable vector nucleic acid prior to packaging into a vector. Thus, the promoter used to direct expression of a heterologous nucleic acid depends on the particular application. The promoter is preferably positioned about the same distance from the heterologous transcription start site as it is from the transcription start site in its natural setting. As is known in the art, however, some variation in this distance can be accommodated without loss of promoter function. Often the promoter used to drive the target nucleic acid is a subgenomic or genomic SFV promoter recognized by replicase, preferably the subgenomic promoter. Suitable promoters include, e.g., those derived from Sindbis virus and SFV. Other promoters include any promoter suitable for driving the expression of a heterologous gene in a host cell.

The Togavirus-packageable vector nucleic acid optionally can be operably linked to a bacterial RNA polymerase promoter for *in vitro* transcription prior to introduction of the vector RNA into packaging cells. Similarly, the genes encoding viral structural proteins required for trans-complementing the Togavirus-packageable vector nucleic acid can be operably linked to a bacterial RNA polymerase promoter for *in vitro* transcription. After *in vitro* transcription, the RNA encoding the structural proteins can be introduced into packaging cells along with the vector RNA. Alternatively, a DNA plasmid encoding the vector nucleic acid can be introduced into a packaging cell for transcription *in vivo*. DNA plasmids suitable for *in vitro* transcription of the vector nucleic acid *in vitro* are known to those skilled in the art. These plasmids are commercially available and include promoters for bacterial RNA polymerases such as SP6, T7, or T3 (*see* Sambrook, *supra*; Ausubel, *supra*). For example, the commercially available SFV1 plasmid contains Semliki Forest virus sequences and a bacterial RNA polymerase promoter (Liljeström & Garoff, *supra*).

Any heterologous nucleic acid that is suitable for introduction into a host cell as the target nucleic acid can be used in the present invention by one skilled in the art. Genes useful for gene therapy can be introduced into mammals using the methods and viruses of this invention. Genes encoding proteins, antisense RNA, and ribozymes are useful in gene therapy for restenosis.

An "antisense" nucleic acid refers to a nucleic acid that is complementary to a target sequence of choice and capable of specifically hybridizing with the target molecules. The term antisense also encompasses a DNA sequence in an expression cassette from which antisense RNA is transcribed. Antisense nucleic acids hybridize to a target polynucleotide and interfere with the transcription, processing, translation or other activity of the target nucleic acid. An antisense nucleic acid can inhibit DNA replication or DNA transcription by, for example, interfering with the attachment of DNA or RNA polymerase to the promoter by binding to a transcriptional initiation site or a template. It can interfere with processing of mRNA, poly(A) addition to mRNA or translation of mRNA by, for example, binding to regions of the RNA transcript such as the ribosome binding site. It can promote inhibitory mechanisms of the cells, such as promoting RNA degradation via RNase action. The inhibitory polynucleotide can bind to the major groove of the duplex DNA to form a triple helical or "triplex" structure. Methods of

inhibition using antisense nucleic acids therefore encompass a number of different approaches to altering expression of specific genes that operate by different mechanisms (*see, e.g.*, Helene & Toulme, *Biochim. Biophys. Acta.*, 1049:99-125 (1990)).

Ribozymes are catalytic RNA molecules that cleave other RNA molecules having particular nucleic acid sequences. General methods for the construction of ribozymes, including hairpin ribozymes, hammerhead ribozymes, RNase P ribozymes (i.e., ribozymes derived from the naturally occurring RNase P ribozyme from prokaryotes or eukaryotes) are known in the art (*see, e.g.*, Castanotto *et al.*, *Advances in Pharmacology* 25:289-317 (1994) (providing an overview of ribozymes in general, including group I ribozymes, hammerhead ribozymes, hairpin ribozymes, RNase P, and axhead ribozymes)).

Suitable classes of target nucleic acids include suicide genes, transdominant inhibitors of proteins, ribozymes, antisense RNA, and genes encoding products that enhance re-endothelialization. In one preferred embodiment, proto-oncogenes have been associated with vascular smooth muscle cell growth and cell cycle control. Specifically, proto-oncogenes such as c-myc, c-myb, c-jun, c-fos, and c-ras have been linked to vascular hypertrophy. Inhibitors of these proteins are therefore preferred target nucleic acids, e.g., transdominant proteins or antisense/ribozyme molecules that inhibit the proto-oncogenes. Inhibitors of the proto-oncogene c-myc represent a particularly preferred embodiment of a target nucleic acid, since expression of c-myc reaches its peak as early as 2 hours after balloon injury (Bennett *et al.*, *J. Clin. Invest.* 93:820-828 (1994)). The target nucleic acid can therefore encode antisense molecule or ribozyme that inhibits c-myc expression.

In another preferred embodiment, viral suicide genes that convert compounds into toxins for dividing cells have been shown to alleviate vascular hypertrophy. The gene encoding herpes simplex virus thymidine kinase combined with ganciclovir treatment is a preferred embodiment of a suitable suicide gene as a target nucleic acid. Another preferred suicide gene is cytosine deaminase.

In another preferred embodiment, inhibitors of the components of the renin-angiotensin system (RAS) can be used as target nucleic acids. The renin-angiotensin system is implicated in many cardiovascular diseases, such as hypertension, myocardial infarction, restenosis, atherosclerosis, and cardiomyopathy. Angiotensin II

(Ang II), the octapeptide bioactive end product of the RAS system, is cleaved from angiotensin I (Ang I), by angiotensin-converting enzyme (ACE) and/or human chymase. Ang I is formed from the prohormone angiotensiogen by renin. Ang II induces vasoconstriction, cardiovascular muscle cell growth, collagen production, and has antidiuretic properties. Ang-(1-7) and Ang-(1-9) are formed from Ang I and are endogenous RAS inhibitors. Ang-(1-7) and Ang-(1-9) have been shown to have opposite effects to Ang II. Ang II receptor angonists and Ang II antipeptide are also suitable inhibitors of the RAS system. Therefore, preferred embodiments of target nucleic acids include inhibitors of ACE, human chymase, renin, angiotensinogen, the signal peptide of angiotensinogen, Ang I, and Ang II. Other preferred embodiments of target nucleic acids that inhibit the RAS system include nucleic acids encoding Ang-(1-7), Ang-(1-9), Ang II antipeptide, and Ang II receptor peptide agonists: (Sar^7, Ala^8) Ang II, (Sar^7, Gly^8) Ang II, (Sar^7, Ile^8) Ang II, (Sar^7, Leu^8) Ang II, (Sar^7, Thr^8) Ang II, and (Sar^7, Val^5, Ala^8) Ang II (saralasin).

Another preferred embodiment of target nucleic acids are endothelial relaxing factors. Relaxing factors are produced by the endothelium and are potentially important inhibitors of atherosclerosis, restenosis, hypertension, and development of heart failure. Therefore, the genes for these factors provide preferred embodiments of target nucleic acids: constitutive endothelial nitric oxide synthase (ec-NOS), bradykinin, and ($Ala^{2,6}$, des-Pro^3) bradykinin (which is a potent ACE inhibitor).

Another preferred embodiment of target nucleic acids are inhibitors of non-endothelial cell growth factors. Ang II induces the growth of vascular and cardiac smooth muscle cells. It has been shown that other growth factors are released upon stimulation with Ang II and play a role in non-endothelial cell growth. Preferred embodiments of target nucleic acids therefore include inhibitors of platelet derived growth factor A (PDGF-A), fibroblast growth factor (FGF) and transforming growth factor-β (TGF-β). Gene products that enhance re-endothelialization are also useful target nucleic acids, e.g., VEGF.

Examples of target nucleic acids therefore include inhibitors of: c-myc, c-myb, c-jun, c-fos, c-ras, p21, Rb, p16, ICAM, NF-KB, VCAM-1, CAM, ACE, human chymase, angiotensinogen, Ang I, Ang II, angiotensinogen signal peptide, renin, PDGF-A, FGF, and TGF-β. Additional examples of target nucleic acids include nucleic acids

encoding: constitutive endothelial nitric oxide synthase (ec-NOS), bradykinin, ($Ala^{2,6}$, des-Pro^{3}) bradykinin, VEGF, Ang-(1-7), Ang-(1-9), Ang II antipeptide, and Ang II receptor peptide agonists: (Sar^{7}, Ala^{8}) Ang II, (Sar^{7}, Gly^{8}) Ang II, (Sar^{7}, Ile^{8}) Ang II, (Sar^{7}, Leu^{8}) Ang II, (Sar^{7}, Thr^{8}) Ang II, and (Sar^{7}, Val^{5}, Ala^{8}) Ang II (saralasin). Additional target nucleic acids for controlling smooth muscle proliferation include the use of suicide genes such as the genes encoding herpes simplex virus thymidine kinase with ganciclovir treatment (*see, e.g.*, WO 95/25807); bacterial cytosine deaminase with 5FC, and β-glucosidase. Genes encoding selectable markers, such as those that confer antibiotic resistance, can also be used as an assay to detect packaging of the Togavirus-packageable vector nucleic acid by packaging cells into vectors.

C. Packaging of the vector nucleic acid

The Togavirus-packageable vector nucleic acid or vector nucleic acid can be packaged by any means suitable in the art (*see* Sambrook, *supra*; Ausubel, *supra*; *see also* USSN 08/454,641, *supra*). In one embodiment, the Togavirus-packageable vector nucleic acid is introduced into packaging cells by standard methodology, e.g., transfection, electroporation and the like. The vector can be provided as a plasmid, which is transcribed in the packaging cell, or transcribed *in vitro* and then introduced to the cell as RNA. In another embodiment, a vector nucleic acid that lacks a Togavirus packaging site can be packaged into the vector by standard methodology, e.g., by forming a lipid:nucleic acid complex.

In one embodiment, the Togavirus-packageable vector nucleic acid is typically packaged by trans-complementing cells called packaging cells. The packaging cells can either transiently express the packaging components, by co-introduction of the vector nucleic acid with a plasmid or RNA encoding structural genes for the viral particle, or stable packaging cell lines can be produced.

In one embodiment, both the vector nucleic acid and the genes encoding viral structural proteins are transcribed *in vitro*. The vector RNA has a viral packaging site, while the RNA encoding the viral structural proteins lacks a viral packaging site. The RNAs are introduced into the packaging cell, e.g., via electroporation. Replicase is translated from the vector RNA. The replicase then amplifies the RNA encoding the viral structural proteins, which can have a promoter recognized by replicase. The viral

structural proteins are then translated. These proteins recognize the viral packaging signal on the vector RNA and package the vector RNA into a viral particle.

Two trans-complementing helper plasmids for SFV are called Helper-1 and Helper-2 (*see, e.g.*, Berglund *et al.*, *Biotechnology* 11:916-920 (1993)). The Helper-1 packaging vector includes the structural proteins for nucleocapsid formation but lacks a packaging site. The Helper-2 packaging vector is a variant of Helper-1 in which the SFV viral p62 glycoprotein is mutated. Wild type p62 is normally cleaved into its active mature "E2" form by a host cell endoprotease. Mutant p62 cannot be cleaved *in vivo* and must be cleaved *in vitro* by chymotrypsin to produce infective particles. This mutation provides an additional biosafety measure.

Stable packaging cell lines are made by stably or transiently transducing a mammalian cell with a packaging plasmid, most preferably by transducing a human cell. The transduction of mammalian (including human) cells is known in the art. Host cells are competent or rendered competent for transformation by various known means. There are several well known methods of introducing DNA into animal cells. These include: calcium phosphate precipitation, fusion of the recipient cells with bacterial protoplasts containing the DNA, treatment of the recipient cells with liposomes containing the DNA, DEAE dextran, receptor-mediated endocytosis, electroporation and micro-injection of the DNA directly into the cells.

The culture of cells used in conjunction with the present invention, including cell lines and cultured cells from tissue or blood samples is well known in the art (Freshney, *Culture of Animal Cells, a Manual of Basic Technique* (3d ed., 1994)) and the references cited therein provides a general guide to the culture of cells. Transformed cells are cultured by means well known in the art (*see* Kuchler *et al.*, *Biochemical Methods in Cell Culture and Virology* (1977)). Mammalian cell systems often will be in the form of monolayers of cells, although mammalian cell suspensions are also used. Illustrative examples of mammalian cell lines include VERO and HeLa cells, 293 embryonic kidney cells, Chinese hamster ovary (CHO) cell lines, W138, BHK, Cos-7 or MDCK cell lines (*see, e.g.*, Freshney, *supra*). Human cells are most preferred.

D. Production of viral stocks

Suitable methods know to those skilled in the art are used to harvest the packaged vector nucleic acid from the packaging cell lines. Generally, after introduction of the Togavirus vector, the packaging cells are incubated under standard cell culture conditions to allow packaging of the vector nucleic acid and budding of viral particles into the cell supernatant. The cell supernatant is then collected after a suitable amount of time, *e.g.*, after 24 hours. The supernatant is then frozen or used immediately. Components from the cell supernatants can be further purified using standard techniques. For example, Togavirus particles in the supernatant can be purified from the supernatant by methods typically used for viral purification such as centrifugation, chromatography, affinity purification procedures, and the like.

E. Characterization of Togavirus vectors and assay for transduction

Togaviral particles and Togavirus vectors can be characterized by any of a number of means well known to those of skill in the art, to ensure that they carry the vector nucleic acid. These methods include the detection of specific vector RNA by well known methods such as northern analysis, dot blot analysis, gel electrophoresis, PCR, and RNase protection assays, as well as detection of protein expressed by a target nucleic acid.

The biological activity of the Togavirus particles and vectors can be tested by infecting any suitable cell type and examining vector nucleic acid levels, and RNA and protein expression of the target nucleic acid. These methods can also be used as an assay to measure the relative transduction efficiency of the Togavirus vectors.

To characterize the vector and assay for transduction efficiency, control cells (typically endothelial cells) and test cells are infected after titering virus-containing cell supernatants according to standard methods. A suitable time after infection, cells are examined for Togavirus and target nucleic acid expression activity. RNA and protein expression from the target nucleic acid or the vector nucleic acid is detected and quantified by any of a number of means well known to those of skill in the art. These include analytic biochemical methods such as spectrophotometry, radiography, electrophoresis, capillary electrophoresis, high performance liquid chromatography (HPLC), thin layer chromatography (TLC), hyperdiffusion chromatography, and the like,

and various immunological methods such as fluid or gel precipitin reactions, immunodiffusion (single or double), immunoelectrophoresis, radioimmunoassays (RIAs), enzyme-linked immunosorbent assays (ELISAs), immunofluorescent assays, and the like. RNA expression can be detected by well known methods such as Northern analysis, gel electrophoresis, RT-PCR (reverse transcriptase PCR), RNase protection, radiolabeling and scintillation counting, and affinity chromatography.

To monitor the progress of infection, a marker or "reporter" gene is optionally encoded by the vector nucleic acid. The inclusion of detectable markers provides a means of monitoring the infection of target cells. Markers include components of the beta-galactosidase gene, the firefly luciferase gene, and the green fluorescence protein (*see, e.g.*, Chalfie *et al.*, *Science* 263:802 (1994)).

The RNA or protein expression of the target nucleic acid, or the level of the vector nucleic acid in the test cell (e.g., a vascular smooth muscle cell or a cardiomyocyte) is compared to expression levels in control cells, typically endothelial cells. This comparison gives a measure of the relative transduction efficiency of the vector for the control v. the test cells.

IV. Transduction of target cells with Togavirus vectors

The Togavirus vectors of the present invention are used in cell transduction procedures for mammalian gene therapy, preferably for human gene therapy. The vectors are particularly useful for gene therapy because they have the ability to selectively transduce specific populations of cells, e.g., non-endothelial cardiovascular cells. The vectors therefore provide gene therapy techniques for combating restenosis. Gene therapy for restenosis can be used to transduce cells with either an *ex vivo* or an *in vivo* procedure.

In addition to the powerful therapeutic uses of the vectors and methods of the present invention, the invention is useful for research. These vectors of the invention can be used to introduce nucleic acids into the host cell to study the activity of these nucleic acids and the proteins that they encode on the host cell.

A. Transduction of cells ex vivo

Ex vivo methods of gene therapy involve transducing a cell *ex vivo* with a Togavirus vector of this invention, and introducing the cell into the organism. Target cells include non-endothelial cells cultured from a patient that can be specifically transduced with a Togavirus vector (*see, e.g.*, Freshney *et al.*, *supra* and the references cited therein for a discussion of how to isolate and culture cells from patients; *see* Wilson *et al.*, *Science* 244:1344-1346 (1989)). Transduced cells can be introduced at the site of a vascular lesion via a catheter or injection. These cells are then capable of producing inhibitory substances encoded by the target nucleic acid introduced by the Togavirus vector. These substances can be used to inhibit the growth of cells such as smooth muscle cells that cause vascular lesions (*see, e.g.*, Wilson *et al.*, *supra*).

Any suitable non-endothelial cardiovascular cell can be transduced *in vitro* for *ex vivo* gene therapy using the method of the invention. Both cardiovascular cells such as smooth muscle cells and cardiac muscle cells, and non-cardiovascular cells such as fibroblasts can be specifically transduced using Togavirus vectors (Huckriede *et al.*, *Eur. J. Biochem.*, 237:288-294 (1996)). Because the Togavirus vector can be engineered to produce a lytic infection, the transduced cells can lyse after delivery to a particular site in the patient, releasing non-endothelial cell inhibitory proteins encoded by the target nucleic acid or growth factors that promote re-endothelialization.

Cells are transduced *in vitro* according to methods known to those skilled in the art as described above, e.g., calcium phosphate precipitation, fusion of the recipient cells with bacterial protoplasts containing the DNA, treatment of the recipient cells with liposomes containing the DNA, DEAE dextran, receptor-mediated endocytosis, electroporation and micro-injection of the DNA directly into the cells. The culture of cells, including cell lines and cultured cells from tissue or blood samples is well known in the art (Freshney, *Culture of Animal Cells, A Manual of Basic Technique* (3d ed. 1994) and the references cited therein).

B. Administration of Togavirus particles and transduced cells

Packaged vector nucleic acid or packaged Togavirus vector nucleic acid (for *in vivo* gene therapy) and transduced cells (for *ex vivo* gene therapy) can be administered directly to a patient, preferably a human. Administration is by any of the

routes normally used for introducing a molecule or cell into ultimate contact with blood or tissue cells. Packaged vector nucleic acid and Togavirus vector nucleic acid is administered in any suitable manner, preferably with pharmaceutically acceptable carriers. Suitable methods of administering such Togavirus vectors and particles in the context of the present invention to a patient are known to those skilled in the art. Often the Togavirus vectors are introduced to the patient at the site of a vascular lesion, for example, during or after a balloon angioplasty procedure. In such example, the vectors or transduced cells are introduced via a catheter that has been placed in the vessel of choice, to deliver the vectors or cells to the site of the lesion (for a description of angioplasty techniques, see *Harrison's Principles of Internal Medicine* (Isselbacher *et al.* eds., 13th ed. 1994); for delivery of cells or vectors to a vessel see Nabel *et al.*, *Science* 244:1342-1344 (1989)).

Pharmaceutically acceptable excipients are determined in part by the particular composition being administered, as well as by the particular method used to administer the composition. Accordingly, there is a wide variety of suitable formulations of pharmaceutical compositions of the present invention. Formulations suitable for parenteral administration, such as, for example, intravenous, intramuscular, intradermal, intraperitoneal, and subcutaneous routes, include aqueous and non-aqueous, isotonic sterile injection solutions, which can contain antioxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient, and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives. Intravenous administration is the preferred method of administration.

The dose administered to a patient, in the context of the present invention should be sufficient to effect a beneficial therapeutic response in the patient over time. The dose will be determined by the efficacy of the particular target nucleic acid in the vector nucleic acid and the condition of the patient, as well as the body weight or surface area of the patient to be treated. The size of the dose also will be determined by the existence, nature, and extent of any adverse side-effects that accompany the administration of a particular Togavirus particle, vector, or transduced cell type in a particular patient.

For administration, Togaviral particles, vectors, and transduced cells of the present invention can be administered at a rate determined by the transduced cell type, and the side-effects of the vector or cell type at various concentrations, as applied to the mass and overall health of the patient. Administration can be accomplished via single or divided doses. For a typical 70 kg patient, a dose equivalent to approximately .1μg to 10 mg are administered. Transduced cells are optionally prepared for reinfusion according to established methods (*see, e.g.*, Abrahamsen *et al.*, *J. Clin. Apheresis* 6:48-53 (1991); Carter *et al.*, *J. Clin. Apheresis* 4:113-117 (1988); and Aebersold *et al.*, *J. Immunol. Methods* 112:1-7 (1988); *see also Remington's Pharmaceutical Science* (Gennaro *et al.*, eds., 17th ed.)).

V. Restenosis inhibitors and measuring inhibition

Approximately 20-30% of patients exhibit restenosis within 6 months of balloon angioplasty. As described above, a wide variety of target nucleic acids are suitable for delivery to non-endothelial cells to inhibit restenosis. Such target nucleic acids typically encode, e.g., proteins, antisense molecules, and ribozymes that inhibit smooth muscle cell proliferation.

Targets for controlling smooth muscle proliferation leading to restenosis include those discussed above, e.g., angiotensin converting enzyme inhibitors (ACE inhibitors); inhibition of cell cycle control proteins such as c-myc (a probable cell cycle control protein); suicide genes, and gene products that enhance re-endothelialization such as VEGF.

The ability of a target nucleic acid to inhibit restenosis can be tested *in vitro*. Such tests are performed by transducing a dividing test cell *in vitro* with a Togavirus vector expressing the target nucleic acid of choice. Inhibition of restenosis is measured by comparing the proliferation or death of the control cell to a test cell. Relative proliferation is measured by standard means in the art, e.g., radionucleotide uptake experiments (*see generally* Ausubel *et al.*, *supra*; Sambrook *et al.*, *supra*). Cell death is measured by standard means in the art, e.g., trypan blue exclusion. The test cell is compared to a control cell that has not been treated with the restenosis inhibitor. Inhibition of restenosis can be measured either by cessation of cell division, by killing the test cell, or other suitable means. Inhibition of restenosis is achieved with the *in vitro* test

if relative to the control cell the test cell is inhibited 20%, preferable 40%, more preferably 60%.

The ability of a target nucleic acid to inhibit restenosis can also be measured *in vivo* in a patient. Typically, restenosis is indicated by a reoccurrence of angina pectoris that is accompanied by evidence of ischemia in an exercise test (*see, e.g., Harrison's Principles of Internal Medicine, supra*). Patients who have been administered the vectors of the invention can be examined to determine whether the target nucleic acid inhibits restenosis. Such patients are compared to a control group of patients who have either experienced restenosis or who have not had a reoccurrence of restenosis. Inhibition of restenosis is measured by performing an exercise test accompanied by a concurrent EKG. Specific changes in the EKG indicate whether restenosis has occurred (*see Harrison's, supra*). Restenosis can be confirmed or excluded by coronary angiography, however, angiography is not the preferred initial test to determine restenosis inhibition in this example due to risks associated with the procedure. Inhibition of restenosis is arbitrarily defined for the purposes of this example. Patients in the control group who have not experienced restenosis are assigned a heart function value of 100. Patients in the control group who have experienced restenosis are assigned a heart function value of 0. Inhibition of restenosis is achieved when the test patients exhibit a heart function value, based on the exercise EKG, that is about 20 on the relative scale of 0-11, preferable above 40, more preferable above 60 as compared to patients in the test group.

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to one of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

EXAMPLES

The following examples are provided by way of illustration only and not by way of limitation. Those of skill in the art will readily recognize a variety of noncritical parameters that could be changed or modified to yield essentially similar results.

Methods

A. Isolation and culture of vascular smooth muscle cells

A7r5 rat embryonic aortic smooth muscle cell lines (ATCC, CRL-1444, Rockville, USA) were a kind gift from Dr. H. de Smedt (Laboratory for Physiology, Catholic University of Leuven, Belgium) and were grown in Dulbecco's Modified Eagle's Medium (DMEM) (ICN, Zoetermeer, The Netherlands) supplemented with 50 units/ml penicillin/streptomycin (Life Technologies, Breda, The Netherlands), 10 mM HEPES (Life Technologies) and 10% Fetal Calf Serum (FCS) (Life Technologies). HA-VSMC human aortic smooth muscle cell lines (ATCC; CRL-1999) were grown on nutrient mix F12 Kaighn's modification (Life Technologies) supplemented with 2 mM L-glutamine, 10 mM HEPES, 1x ITS-X supplement (Life Technologies), 10 mM TES (Sigma, Deisenhofen, Germany), 50 μg/ml ascorbic acid (Sigma) and 30 μg/ml endothelial cell growth supplement (ICN). Primary cultured human umbilical cord vascular smooth muscle cells (hVSMC) were isolated and cultured as described before (Jaffe *et al., J. Clin. Invest.* 52:2745-2756 (1973)). All cells were maintained in culture at 37°C and 5% CO_2.

B. Isolation and culture of endothelial cells

Bovine aortic endothelial cells (BAEC) were isolated and cultured as previously described (Paul *et al., Hypertension* 25:683-693 (1995)). The human endothelial cell line ECV 304 (ECACC, Salisbury, UK) was cultured in M 199 containing 10% FCS. The human endothelial cell line EA.hy 926 (a kind gift from Dr. C.-J. Edgell, University of North Carolina, Durham, N.C., USA to M. Paul) was grown in DMEM supplemented with 10% FCS. EC-RF24 (Paul *et al., Hypertension* 25:683-693 (1995)) (a kind gift from Prof. Dr. H. Pannekoek, Amsterdam Medical Centre, Amsterdam, The Netherlands to Y. Pinto) were grown on a 1:1 mixture of M 199 and RPMI 1640 (Life Technologies) supplemented with 2 mM glutamin, 50 units/ml

penicillin/streptomycin and 20% heat-inactivated human serum. Human umbilical vein endothelial cells (HUVEC) (a kind gift of Dr. V.J.J. Bom, Dpt. of Haematology, Acadamic Hospital Groningen The Netherlands) were isolated according to a modified protocol of Jaffe *et al.* (Jaffe *et al., J. Clin. Invest.* 52:2745-2756 (1973)) as described elsewhere (Mulder *et al., Thromb. Res.* 80:399-411). Endothelial cells were grown on collagen A-coated (Biochrom) plastic culture wells or flasks. All cells were kept in culture at 37°C and 5% CO_2.

C. Isolation and culture of cardiac myocytes

Cardiac myocytes (rCM) were isolated from neonatal rats and cultured as described elsewhere (Webster *et al., J. Biol. Chem.* 268:16852-16858 (1993)) on collagen-coated plastic slides (Nunc). After 48 h, the cells were rinsed with serum free medium and exposed to virus.

D. Virus production

Ad5LacZ viral stocks were produced and quantified using HEK 293 cells (Microbix Biosystems Inc., Toronto, Canada). The stock solution was determined to contain 10^7 Biosystems Inc., $pfu \cdot ml^{-1}$. pSFV3-LacZ was produced using pSFV-Helper1, to obtain pSFV3-LacZ/h1, or Helper-2, to obtain pSFV3-LacZ/h2, and quantified using BHK21 cells as described previously (Liljeström *et al., Biotechnology* 9:1356-1361 (1991)). Titers were between 10^7 and 10^8 infectious units per ml. In all cases, the virus stocks that were used originated from the same virus batch.

E. Efficiency and timing of expression of pSFV3-LacZ/h1 and Ad5LacZ

24-well clusters (Nunc, Roskilde, Denmark) were seeded with 25,000 VSMC or 50,000 EC per well. VSMC and EC were allowed to regain morphology for 24 and 48 h, respectively. After this, the growth medium was taken off, the cells were washed with 1x phosphate buffered saline (PBS) (Blochrom) and the virus was added, as described below. The virus was allowed to bind for 1 h, unless indicated otherwise. After this, the cells were washed with PBS and growth medium was added. In every experiment, negative controls were included, i.e. cells that were not exposed to the virus but to serum-free medium.

In order to estimate the number of infectious units, dilution ranges (10 - 10^{10} fold dilutions) of virus stocks were prepared using serum-free DMEM. 200 μl of virus dilutions were added to separate wells containing VSMC (both pSFV3-LacZ/h1 and Ad5LacZ) and EC (pSFV3-LacZ/h1). After binding of the virus, the cells were cultured for 24 h (pSFV-LacZ) or 72 h (Ad5LacZ).

pSFV was compared to Ad5LacZ in subconfluent cultures of EA.hy 926 cells in a six-well cluster, using 400 μl of each virus stock per well and allowing 48 h of viral gene expression, at 37°C, 5% $C0_2$. During viral gene expression, cells were kept in growth medium.

To estimate the time course of pSFV3-LacZ/h1 and Ad5LacZ expression, 12,500 A7r5 cells/well were seeded in 48-well clusters. 200 μl of virus dilution was added per well. After incubation with the virus, the cells were cultured for 1, 3, 6, 12, 24, 48 and 72 h, respectively. The procedures were repeated in a separate experiment for HA-VSMC.

To investigate the possibility of gene transfer to cardiovascular cells other than VSMC, neonatal cardiac myocytes were cultured. These cardiac myocytes were incubated with 50 μl of pSFV3-LacZ/h1 stock solution. After 14 h, 200 μl of growth medium was added, and 22 h after addition of the virus, cells were fixed and analyzed as described below.

F. Efficiency and cytotoxicity of pSFV3-LacZ/h2 and Ad5LacZ.

A7r5 and HUVEC were seeded in 24-well clusters and treated with virus as described above. At 1, 2, 3, 6 and 12 days after exposure to the virus, the culture medium was collected, the cells were detached from the wells by trypsinization and resuspended in the collected medium. At each time point, the number of viable cells was counted in suspensions obtained from one well per treatment by Trypan Blue exclusion in a Bürker counting chamber. Thus, each value represents the mean ($\pm$SEM) obtained from 6 samples from one well. Additionally, the effect of pSFV3-LacZ/h2 on survival of HUVECs after 12 days was assessed. Furthermore, affinity of pSFV3-LacZ/h2 and Ad5LacZ for A7r5 and HUVEC were compared.

G. Histological procedures

After the expression times indicated above, the cells were washed with PBS and fixed with 1.25% glutaric aldehyde (Sigma) for 5 min. at room temperature. Thereafter, the cells were washed with PBS and incubated with X-Gal staining fluid for 1-4 h at 37 °C. X-Gal staining fluid contained 1 mg·ml^{-1} 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (Eurogentec, Seraing, Belgium), 5 mM $K_3Fe(CN)_6$ (Merck, Amsterdam, Holland), 5 mM $K_4Fe(CN)_6$ (Merck), 2mM $MgCl_2 \cdot 6H_2O$ (Merck) and 4% N,N-dimethylformamide (Sigma). Finally, the cells were washed with PBS, post-fixed with 1,25% glutaric aldehyde for 2 min. and either analyzed immediately or stored under 90% glycerol (Jansse, Beersse, Belgium) until analysis.

H. Comparison of virus titers and efficiency of gene transfer

To quantify to the efficiency of gene transfer, the percentage of LacZ-positive cells was determined. After the staining procedure, treated and control cultures were analyzed *in situ* under a light microscope at random locations (magnifications: 63x and 200x). If necessary, photographs were taken from random locations within the separate wells and used for analysis. Each value represents the mean (±SEM) of five random samples in a single well. Data from a single experiment that were obtained from both direct observation under a light microscope and photographical analysis were similar (data not shown).

To estimate virus titers, the dilution step just before the one resulting in no LacZ expression was used. The number of positive cells in the wells which received this virus dilution were counted. The titers were calculated as $N_p * I/DF * 5$ and expressed in infectious units per ml (IU·ml^{-1}), where N_p = number of positive cells, DF = dilution factor. One IU corresponds to the amount of virus capable of transferring LacZ to a single cell.

I. Statistical procedures

All means were compared with t-tests for independent samples. The viability curves for A7r5 were analyzed and compared with ANOVA for repeated measures.

Example 1: Efficiency of viral gene transfer into vascular smooth muscle cell, endothelial cells, and cardiomyocytes

In the present study recombinant SFV was compared to adenovirus for gene transfer into cardiovascular cells. The results show that SFV efficiently transfers the LacZ gene to vascular smooth muscle cells and cardiomyocytes. In contrast to the adenovirus vector Ad5LacZ, pSFV3-LacZ does not cause efficient gene transfer into endothelial cells.

A. Vascular smooth muscle cells

Table 1 shows adenovirus and SFV titers determined in VSMC. The results show that the titer depended on the cell type and that there is no clear difference between both viruses. Both viruses were equally able to transfer LacZ to all three types of VSMC. After gene transfer with Ad5LacZ the percentage of LacZ-positive HA-VSMC is significantly higher than that obtained with pSFV3-LacZ/h1 ($p < 0.05$). Negative controls did not contain blue stained cells.

B. Endothelial cells

The number of LacZ-positive BAEC, ECV 304 and EA.hy 926 cells after gene transfer with pSFV3-LacZ/h1 never exceeded 2.6% while HA-VSMC, as a positive control, always showed a high efficiency of gene transfer. This result is also reflected by the titration experiment shown in Table 2. As it is known that a small percentage of primary cultured BAEC are of other origin (e.g., smooth muscle cells or fibroblasts), this could result in a higher titer for BAEC as compared to the other EC. Still, the maximal percentage of positive BAEC is not higher than that of EA.hy 926. In a separate experiment EC-RF24 was tested, yielding similar results. In contrast to pSFV3-LacZ/h1, Ad5LacZ transferred LacZ into EA.hy 926 with moderate efficiency (pSFV3-LacZ/h1, $1.1\% \pm 0.2$ vs. Ad5LacZ, $16.7\% \pm 3.1$. $p=0.007$). Comparison of pSFV3-LacZ/h2 with Ad5lacZ in HUVEC resulted in a similar observation ($1.0\% \pm 0.4$ vs. $33.6\% \pm 8.3$). Medium-treated culture were negative.

C. Cardiac myocytes

The amount of LacZ-positive cardiac myocytes after gene transfer with pSFV3-LacZ/h1 was 79% ± 1. The morphology of positive myocytes after 22 h of expression showed that cells were intact.

The results show that SFV efficiently transfers LacZ to VSMC and rCM. In contrast to Ad5LacZ, pSFV3-LacZ does not cause efficient gene transfer into EC. Therefore, pSFV3-LacZ can be considered as a vector with relative specificity for non-endothelial cells. Sensitivity of both EC and VSMC for adenoviral vectors *in vitro* and *in vivo* has been described extensively by others (Rome *et al.*, *Arterioscler. Thromb.* 14:148-161 (1994); Steg *et al.*, *Circulation* 90:1648-1656 (1994); Zhou *et al.*, *Chin. Med. J. Engl.* 108:493-496 (1995)) and confirmed in the present study. Selective adenovirus-mediated gene transfer to EC has been shown to be feasible (Schulick *et al.*, *Circ. Res.* 77:475-485 (1995)).

Table 1: Virus titer estimations of pSFV3-LacZ/h1 and Ad5LacZ in vascular smooth muscle cells, expressed in infectious units per ml (IU·ml). There is no obvious difference in virus titers.

Virus titers for VSMC		
cell type	*pSFV3-LacZ/hl*	*Ad5LacZ*
HA-VSMC	$4x10^5$ IU·ml^{-1}	$2x10^5$ IU·ml^{-1}
A7r5	$5x10^5$ IU·ml^{-1}	$5x10^4$ IU·ml^{-1}
hVSMC	$2x10^5$ IU·ml^{-1}	$1.6x10^5$ IU·ml^{-1}

Table 2: Virus titer determination in endothelial cells (BAEC, ECV 304, EA.hy 926, EC-RF24) and human vascular smooth muscle cells (HA-VSMC), expressed in infectious units per ml (IU·ml). Titers for endothelial cells are obviously lower than for smooth muscle cells.

pSFV3-LacZ/h1 titers for endothelial cells	
cell type	*virus titer*
BAEC	3000 $IU \cdot ml^{-1}$
ECV 304	450 $IU \cdot ml^{-1}$
EA.hy 926	200 $IU \cdot ml^{-1}$
EC-RF24	250 $IU \cdot ml^{-1}$
HA-VSMC	$2x10^4$ $IU \cdot ml^{-1}$

Example 2: Time course of viral expression

The adenovirus and SFV vectors were used to examine the time course of viral gene expression in non-endothelial cardiovascular cells. In a time course experiment with A7r5 rat embryonic aortic smooth muscle cells, the first positive cells were seen 1 h after incubation with pSFV-LacZ/h1 (31%) and 6 h after incubation with Ad5LacZ (<1%). In addition, there was an increasing intensity of LacZ staining in tune. At each time point, the differences between efficiency of gene transfer by pSFV3-LacZ/h1 and Ad5LacZ were significant ($p \leq 0.05$). Medium-treated cultures were negative.

In HA-VSMC (vascular smooth muscle cells), initial LacZ expression was seen after 6 h for pSFV3-LacZ/h1 (22.5% ±7.8) and after 12 h for Ad5LacZ (34.0% ±5.6). Maximal efficiency was reached after 24 h for pSFV3-LacZ/h1 (78.4% ±6.8) and 48 h for Ad5LacZ (54.5%±5.2). Due to low background staining that occurred after long exposure of HA-VSMC to LacZ staining solution at 1 h and 3 h, low expression could be masked. Therefore, expression earlier than 6 h cannot be excluded for pSFV3-LacZ and 12 h for Ad5LacZ.

Example 3: Cytotoxicity of adenovirus vectors and SFV vectors

The effect of pSFV3-LacZ/h2 and Ad5LacZ on growth of A7r5 smooth muscle cells was examined. Both viruses cause cell death and after 12 days the amount of A7r5 was too low to be counted. In HUVEC endothelial cells, however, the number of cells was not decreased 12 days after exposure to pSFV3-LacZ/h2 ($3.6x10^4 \pm 3739$ cells/well at day 0 vs. $5.7x10^4 \pm 3867$ cells/well at day 12) or vehicle ($4.4x10^4 \pm 2656$ cells/well at day 0 vs. $4.6x10^4 \pm 4110$ cells/well at day 12). The percentage transfection efficiency reached in A7r5 was higher than 80%. However, it was observed that A7r5 surviving the period of 12 days were not expressing LacZ.

Both pSFV3-LacZ and Ad5LacZ are cytotoxic to VSMC, whereas HUVEC survive exposure to pSFV3-LacZ. pSFV3-LacZ will cause inhibition of target cell protein synthesis, ultimately leading to apoptosis of the transfected cells (Frolov *et al. Proc. Natl. Acad. Sci. USA* 93:11371-11377 (1996); Strauss *et al. Microbiol. Rev.* 58:491-562 (1994)). Recently, it has been shown that overexpression of the proto-oncogene bcl-2 allowed survival of rat prostatic adenocarcinoma cells after exposure to SFV (Scallan *et al., J. Virol.* 71:1583-1590 (1997)). Others have shown that Ad5LacZ is cytotoxic to both EC (Schulick *et al., Circ. Res.* 77:475-485 (1995)) and VSMC (Schulick *et al., Circulation* 91:2407-2414 (1995)) *in vivo*.

Example 4: Production of Recombinant SFV with Helper-1 and Helper-2 Vectors

Production of recombinant SFV with pSFV-Helper-1 (helper-1), as described above, results in the formation of low amounts of replication-proficient virus (RPV) which might cause an infection (Berglund *et al., Biotechnology* 11:916-920 (1993)). Therefore, a packaging vector called pSFV-Helper-2 (helper-2) was used as described above to produce particles with a mutated P62 spike protein (Berglund *et al. Biotechnology* 11:916-920 (1993)). pSFV-Helper-1 was used to obtain pSFV3-LacZ/h1, and pSV-Helper-2 was used to obtain pSFV3-LacZ/h2 Virus stocks were quantified using BHK21 cells as described previously (Liljeström *et al., Biotechnology* 9:1356-1361 (1991)).

Gene transfer was examined with both Helper-1 and Helper-2 derived SFV vectors. The timing of viral gene expression was examined with Helper-1 derived vectors, and cytotoxicity of Helper-2 derived vectors was assessed. In each case the

Helper-2 virus vectors provided suitable virus stocks and transduction of the non-endothelial cardiovascular cells, as described above.

The wild-type p62 is normally cleaved into its active, mature form E2 by a host endoprotease. Mutant p62, however, cannot be cleaved *in vivo* and has to be converted in E2 *in vitro* by chymotrypsin. Thus, conditionally infectious recombinant particles can be produced that do not contain RPV and do not cause pathological effects nor result in immunity in BALB/C mice (Berglund *et al.*, *Biotechnology* 11:916-920 (1993)). Therefore, Helper-2-based recombinant SFV is a suitable vector for *in vivo* gene transfer into vascular smooth muscle cells.

Example 5: *In vivo* cellular tropism of SFV for vascular smooth muscle cells

A. Introduction

This experiment demonstrates the *in vivo* selectivity of SFV for vascular smooth muscle cells in a rat restenosis model. This model involves partial removal of rat aorta endothelium through a balloon catheter procedure, similar to PTCA treatments in humans. Subsequently, the rat is allowed to recover for various periods of time, after which the aorta segment is clipped and recombinant SFV, containing a marker gene, is introduced for a short period of time. Then the circulation is restored and the next day a specimen of the aorta is taken and frozen to be analyzed for marker gene expression. A high-titer recombinant SFV was used for this experiment, carrying the gene for either luciferase or galactosidase. This experiment demonstrates that SFV-derived vectors, such as SFV particles and SFV-derived virosomes can be used for selective delivery of target nucleic acids such as antisense oligonucleotides to vascular smooth muscle cells, at the site of PTCA treatment.

B. Denudation of rat aorta

The rat aorta denudation procedure involves surgery on small blood vessels. Histologically, it has been established that during the denudation procedure approx. 25% of the local endothelium is removed. This is assessed on the basis of neointima formation. Ultimately a very good survival rate has been achieved, with over 75% of the animals surviving the initial denudation and subsequent transfection procedures.

C. Preparation of high-titer recombinant SFV

Recombinant SFV was produced, purified, and characterized as described above and according to standard procedures. Routinely, titers on the order of 10^9 per ml in the culture medium are achieved. Virus based on the Helper-2 vector was used in the present study. This helper produces conditionally infectious virus that requires activation by chymotrypsin prior to use. The SFV nucleic acid used in this study carries the gene for luciferase or β-galactosidase as a marker. High-titer virus carrying the gene for GFP (green fluorescent protein) was also made, which virus produces brightly fluorescent cells.

D. SFV-mediated transfection of rat aorta in vivo

Two independent experiments were carried out, using SFV-luciferase recombinant virus. Groups of 5 rats were treated to remove the aorta endothelium. After 3 days the relevant segment of the aorta was clipped and exposed for 10 min. to approximately 25 μl of high-titer virus (10^6 particles per μl) in PBS, administered under pressure. Subsequently, the virus was removed and the circulation restored. The next day the animals were sacrificed and small segments of the aorta were removed and frozen. These samples were processed for luciferase determination, involving treatment with Triton X100. Luciferase was determined using standard procedures.

In both experiments, clearcut expression of luciferase was established. The luciferase activity was calibrated against a luciferase standard. The data represent averages of 5 individual animals, expressed as pg luciferase per aorta segment, each luciferase assay being carried out in triplicate (*see*, Figure 1). The data shows that there is considerably more luciferase expression in the denuded aorta as compared to control aorta. This result is consistent with the selectivity of SFV in *in vitro* cell culture systems. In control aortas, in the absence of virus, there was no detectable luciferase activity.

A transfection experiment using high-titer Helper-2-derived SFV-β-gal was also conducted, essentially as described above for the experiments involving SFV-luciferase, except that in two separate groups virus was administered both immediately after denudation and 3 days after denudation. After the animals were sacrificed, small segments of the aorta were stained for β-galactosidase expression according to standard procedures, fixed, embedded and thin sections were examined by regular transmission

microscopy. Clearcut blue staining was observed in multiple cells per section, β-galactosidase expression being confined to the vascular smooth muscle cells. (*see*, Figure 2). Similar results were obtained upon virus administration directly after denudation or 3 days after denudation.

Taken together, these results demonstrate selective *in vivo* SFV-mediated gene delivery to rat aorta smooth muscle cells.

What Is Claimed Is:

1. A method of transducing a selected cell with a target nucleic acid, comprising contacting said selected cell with a vector comprising a vector nucleic acid encoding the target nucleic acid, wherein the selected cell is a non-endothelial cardiovascular cell.

2. The method of claim 1, wherein the vector nucleic acid is a Togavirus-packageable vector nucleic acid.

3. The method of claim 2, wherein the Togavirus is an Alphavirus.

4. The method of claim 2, wherein the Togavirus is a Semliki Forest virus.

5. The method of claim 1, wherein the vector further comprises a Togavirus particle.

6. The method of claim 5, wherein the Togavirus particle is a Semliki Forest virus particle.

7. The method of claim 1, wherein the non-endothelial cell is selected from the group consisting of a vascular smooth muscle cell and a cardiomyocyte.

8. The method of claim 1, wherein transduction of the selected cell is performed *in vivo*.

9. The method of claim 1, wherein transduction of the selected cell is performed *in vivo* following balloon angioplasty.

10. The method of claim 1, wherein transduction o performed *in vivo*, and wherein transduction of the selected cell inhit following balloon angioplasty.

11. The method of claim 1, wherein the target nucl restenosis inhibitor.

12. The method of claim 11, wherein the restenosis from the group consisting of a ribozyme, an antisense RNA, a suicid transdominant inhibitor.

13. The method of claim 11, wherein the restenosis from the group consisting of a c-myc inhibitor, a c-myb inhibitor, an converting enzyme (ACE) inhibitor, a FGF inhibitor, a PDGF inhibi inhibitor.

14. The method of claim 11, wherein the restenosis simplex virus thymidine kinase.

15. The method of claim 1, wherein the vector cor that is cleaved *in vitro* by chymotrypsin to produce a mature E2 prot

16. The method of claim 1, wherein the selected c

17. The method of claim 1, wherein the vector is i patient by local injection at a site in the patient proximal to the selec

18. A method of preferentially transducing selecte nucleic acid *in vivo*, comprising contacting the selected cells and fur selected cells with a recombinant Togavirus vector comprising the ta wherein the selected cells are preferentially transduced by the Togav compared to the non-selected cells.

19. The method of claim 18, wherein the Togavirus is an Alphavirus.

20. The method of claim 18, wherein the Togavirus is a Semliki Forest virus.

21. The method of claim 18, wherein the selected cells are non-endothelial cardiovascular cells and the non-selected cells are endothelial cardiovascular cells.

22. The method of claim 18, wherein the selected cells are non-endothelial cardiovascular cells and the non-selected cells are endothelial cardiovascular cells and wherein the non-endothelial cardiovascular cells are transduced with an efficiency of at least about 50 times the efficiency of transducing the non-endothelial cardiovascular cells.

23. An assay for measuring the relative transduction efficiency of a Togavirus vector comprising a vector nucleic acid for a first test cell compared to a second test cell comprising the steps of:

contacting the first test cell with the Togavirus vector;

contacting the second test cell with the Togavirus vector;

measuring the level of vector nucleic acid in the first and second test cell; and,

comparing the level of vector nucleic acid in the first and second test cell to determine the relative transduction efficiency of the Togavirus vector in the first and second test cells.

24. The assay of claim 23, wherein the Togavirus is a Semliki Forest virus.

25. The assay of claim 23, wherein the vector nucleic acid is a Togavirus-packageable nucleic acid.

26. The assay of claim 23, wherein the level of vector nucleic acid is measured indirectly by measuring the level of expression of an encoded nucleic acid or by measuring the level of an encoded polypeptide.

27. The assay of claim 23, wherein the first test cell is a non-endothelial cardiovascular cell and the second test cell is an endothelial cardiovascular cell.

28. A non-endothelial cardiovascular cell comprising a vector nucleic acid encoding a target nucleic acid.

29. The non-endothelial cardiovascular cell of claim 28, wherein the vector nucleic acid is a Togavirus-packageable vector nucleic acid.

30. The non-endothelial cardiovascular cell of claim 29, wherein the Togavirus is an Alphavirus.

31. The non-endothelial cardiovascular cell of claim 29, wherein the Togavirus is a Semliki Forest virus.

32. The non-endothelial cardiovascular cell of claim 28, wherein the non-endothelial cardiovascular cell is selected from the group consisting of a vascular smooth muscle cell and a cardiomyocyte.

33. The non-endothelial cardiovascular cell of claim 28, wherein the non-endothelial cardiovascular cell is *in vivo*.

34. The non-endothelial cardiovascular cell of claim 28, wherein the non-endothelial cardiovascular cell is *in vitro*.

35. The non-endothelial cardiovascular cell of claim 28, wherein the target nucleic acid encodes a restenosis inhibitor.

36. A vector comprising a vector nucleic acid comprising a target nucleic acid subsequence, which target nucleic acid subsequence encodes a restenosis inhibitor.

37. The vector of claim 36, wherein the vector nucleic acid is a Togavirus-packageable nucleic acid.

38. The vector of claim 36, wherein the restenosis inhibitor is selected from the group consisting of a ribozyme, an antisense RNA, a suicide protein, and a transdominant inhibitor.

39. The vector of claim 36, wherein the restenosis inhibitor is selected from the group consisting of a c-myc inhibitor, a c-myb inhibitor, an angiotensin converting enzyme (ACE) inhibitor, a FGF inhibitor, a PDGF inhibitor, and a TGF-β inhibitor.

40. The vector of claim 36, wherein the restenosis inhibitor is herpes simplex virus thymidine kinase.

41. The vector of claim 37, wherein the Togavirus is an Alphavirus.

42. The vector of claim 37, wherein the Togavirus is a Semliki Forest virus.

43. A kit comprising a container, instructions for practicing the method of claim 1, and a vector comprising a vector nucleic acid.

44. The kit of claim 43, wherein the vector nucleic acid is a Togavirus-packageable nucleic acid.

45. The kit of claim 44, wherein the Togavirus is a Semliki Forest virus.

46. The kit of claim 43, wherein the vector nucleic acid comprises a target nucleic acid subsequence; which target nucleic acid subsequence encodes a restenosis inhibitor.

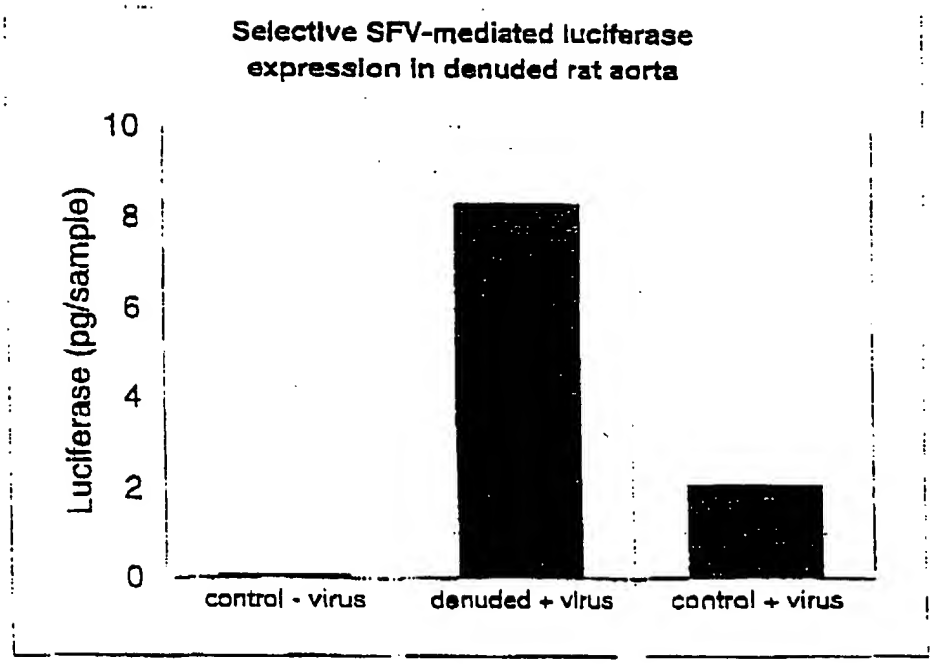
Selective SFV-mediated luciferase expression in denuded rat aorta
Luciferase (pg/sample)
10
8
6
4
2
0
control - virus
denuded + virus
control + virus

FIGURE 1

FIGURE 2A

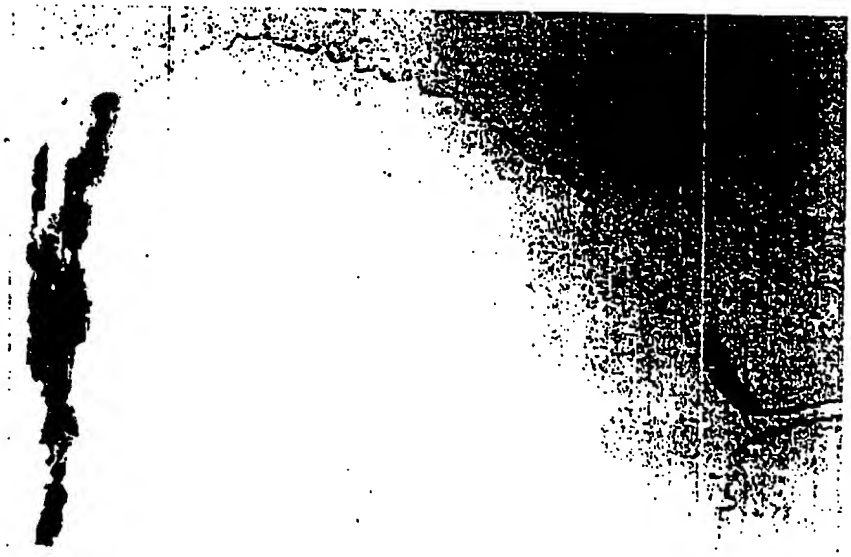

FIGURE 2B

INTERNATIONAL SEARCH REPORT

Int tional Application No
PCT/CA 98/00784

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/86 A61K48/00 C12N5/10 C12Q1/68 G01N33/50

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A61K C12Q G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	OHNO T. ET AL.: "GENE THERAPY FOR VASCULAR SMOOTH MUSCLE CELL PROLIFERATION ALTER ARTERIAL INJURY" SCIENCE, vol. 265, 5 August 1994, pages 781-784, XP000611915 see the whole document ---	1,7-12, 14,16, 17,28, 32-36, 38,40, 43,46
X	BERGLUND P. ET AL.: "Semliki Forest Virus expression system: Production of conditionally infectious recombinant particles." BIOTECHNOLOGY, vol. 11, 1993, pages 916-920, XP002088121 cited in the application	18-20, 23-26, 43-45
A	see the whole document ---	15
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 December 1998	13/01/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Mandl, B

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

In: tional Application No
PCT/CA 98/00784

C.(Continuation) DOCUMENTS CONSIDERED T BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FINKEL T. AND EPSTEIN S. E.: "Gene therapy for vascular disease." FASEB JOURNAL, vol. 9, 1995, pages 843-851, XP002088122 see the whole document	1,7-14, 16,17, 28-36, 43,46
X	WO 95 31541 A (RIBOZYME PHARM INC) 23 November 1995	1-3,5, 7-13,16, 17, 36-39, 41,43, 44,46
Y	see the whole document, especially page 12, lines 6-21	4,6,42, 45
Y	LILJESTROEM P. ET AL.: "A NEW GENERATION OF ANIMAL CELL EXPRESSION VECTORS BASED ON THE SEMLIKI FOREST VIRUS REPLICON" BIO/TECHNOLOGY, vol. 9, December 1991, pages 1356-1361, XP000616021 cited in the application see the whole document	4,6,42, 45
X	WO 96 17072 A (VIAGENE INC) 6 June 1996 see the whole document	1-12, 16-20, 23-26, 36-38, 40-46
P,X	WO 97 38087 A (FROLOV ILYA ;POLO JOHN M (US); VIAGENE INC (US); BELLI BARBARA A ()) 16 October 1997 see page 1, line 1 - page 11, line 23 see page 70, line 18 - page 71, line 28	1-12, 16-38, 41-46
P,X	ROKS, ANTON J. M. ET AL: "A viral gene transfer method for rapid and selective transfection of vascular and cardiac muscle." XIXTH CONGRESS OF THE EUROPEAN SOCIETY OF CARDIOLOGY TOGETHER WITH THE 32ND ANNUAL GENERAL MEETING OF THE ASSOCIATION OF EUROPEAN PAEDIATRIC CARDIOLOGISTS (AEPC), STOCKHOLM, vol. 18, no. SUPPL., 24 - 28 August 1997, page 500 XP002088173 see abstract	1-7,16, 28-32,34

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Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

In tional Application No
PCT/CA 98/00784

C.(Continuation) DOCUMENTS CONSIDERED T BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	ROKS A. J. ET AL.: "Vectors based on Semliki Forest virus for rapid and efficient gene transfer into non-endothelial cardiovascular cells: comparison to adenovirus." CARDIOVASCULAR RESEARCH, vol. 35, no. 3, September 1997, pages 498-504, XP002088123 see the whole document	1-7,16, 28-32,34

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

..ernational application No.
PCT/CA 98/ 00784

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 8-10 and 17-22 and claims 1-7 and 11-16 as far as they refer to an in vivo application are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

In ational Application No
PCT/CA 98/00784

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9531541 A	23-11-1995	US 5646042 A	08-07-1997
		AU 2642295 A	05-12-1995
		EP 0763106 A	19-03-1997
		JP 10500309 T	13-01-1998
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WO 9617072 A	06-06-1996	AU 4594996 A	19-06-1996
		EP 0797679 A	01-10-1997
		US 5814482 A	29-09-1998
		US 5843723 A	01-12-1998
		US 5789245 A	04-08-1998
WO 9738087 A	16-10-1997	AU 2800797 A	29-10-1997